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XENOBIOTIC RELATED INDUCTION OF GENE EXPRESSION

Abstract:

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An isolated nucleic acid molecule comprising a nucleotide sequence encoding a transcriptional enhancer of cytochrome P450 (P450) CYP3A4 production or expression, and uses of the nucleic acid molecule for screening compounds for xenobiotic induction of CYP3A4 expression in cells and animals. Data supplied from the esp@cenet database - Worldwide d71

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Xenobiotic related induction of gene expression

Technical Field

The present invention relates to modulating or effecting gene expression and/or formation of human and non-human animal cytochrome P450 CYP3A subfamily enzymes.

Background Art

Development of therapeutic drugs for medical and veterinary use is important in order to improve and advance treatment modalities in humans and animals. Unfortunately, many drugs that are developed and subsequently used have been found to have limited half lives *in vivo* due to rapid metabolism by the recipient. It would be advantageous to have a system for screening potential new drugs for susceptibility to metabolic action in patients.

A cytochrome P450 (P450) termed CYP3A4 is an important human gene that codes for an enzyme expressed in the liver, and to a lesser extent a range of other tissues. It is quantitatively the most abundant P450 in human liver. The CYP3A4 enzyme is pivotal to the metabolism of many exogenous chemicals (xenobiotics), including therapeutic drugs, as well as a range of endogenous compounds, such as steroid hormones. Changes in the level of expression of CYP3A4 can dramatically affect the rate of elimination of therapeutic drugs and thus impact on their effectiveness. A range of exogenous chemicals, including some therapeutic drugs, (hereafter termed 'xenobiotic inducers') are able to increase the transcriptional rate of the CYP3A4 gene and hence the formation of the CYP3A4 enzyme. The result is the elimination of drugs metabolised by CYP3A4 being greatly increased thereby diminishing their therapeutic effect.

The present inventors have obtained a DNA molecule, lying approximately 7.5 kilobases 5' to the transcription initiation site of the human P450 CYP3A4 gene, that is responsible for the transcriptional induction of the CYP3A4 gene by xenobiotic inducers, including therapeutic drugs and is also involved in the constitutive expression of this gene. This nucleic acid molecule, called a 'xenobiotic-responsive enhancer module' (XREM) by the present inventors, has a number of beneficial uses.

Disclosure of Invention

In a first aspect, the present invention consists in an isolated nucleic acid molecule including a nucleotide sequence forming a transcriptional enhancer of cytochrome P450 (P450) CYP3A4 production or expression.

5 Preferably, the isolated nucleic acid molecule includes a nucleotide sequence substantially as shown in Figure 1 (SEQ ID NO: 1), or a functionally equivalent nucleotide sequence or portion thereof encoding an enhancer of CYP3A4, or a sequence which hybridises to the nucleotide sequence of Figure 1 (SEQ ID NO: 1), or a sequence which shows at least
10 60% homology with the nucleotide sequence of Figure 1 (SEQ ID NO: 1). More preferably, the nucleic acid molecule has at least 80% homology with the nucleotide sequence of Figure 1 (SEQ ID NO: 1) and most preferably the nucleic acid molecule has at least 90% homology with that sequence.

In a preferred embodiment, the present invention consists in an
15 isolated nucleic acid molecule including nuclear receptor response elements from the 5'-flanking region of CYP3A4. Preferably, the response elements are selected from

XREM-DR3-1 GAA TGAACCTTGC TGACCC TCT (SEQ ID NO: 2);
XREM-ER6 CCT TGAAAT CATGTC GGTTC AAGC (SEQ ID NO: 3);
20 XREM-DR6 AGG TGAATC ACAAGC TGAACCT TCT (SEQ ID NO: 4);
XREM-DR3-2 ATA TATTGT TAT TGAACCT ATC (SEQ ID NO: 5); and
Prox-ER6 ATA TGAACCT CAAAGG AGGTCA GTG (SEQ ID NO: 6).

As a number of specific response elements have been identified in the enhancer of CYP3A4 by the present inventors, it will be appreciated that SEQ
25 ID NO: 1 can be used to identify other response elements. As the full sequence of the enhancer is not necessary for subsequent use, the present invention includes within its scope the use of response elements from the enhancer with intermediate or connecting sequences from other sources.

30 Preferably, the isolated nucleic acid molecule has a nucleotide sequence substantially as shown in Figure 1 (SEQ ID NO: 1).

In a preferred form, the CYP3A4 is human CYP3A4. It will be appreciated, however, that the present invention also includes other human CYP3A subfamily enzymes and CYP3A subfamily enzymes from non-human animals.

35 The induction of CYP3A4 is preferably by one or more xenobiotic inducers.

The present invention also includes polynucleotides which hybridise to the sequence shown in Figure 1 (SEQ ID NO: 1) . Preferably, the polynucleotide hybridises to the sequence set out in Figure 1 (SEQ ID NO: 1) under high stringency. As used herein, stringent conditions are those that (a) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄ at 50°C; (b) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (c) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42°C in 0.2 x SSC and 0.1% SDS.

In a further preferred embodiment of the first aspect of the present invention, the polynucleotide capable of hybridising to the nucleotide sequence of Figure 1 (SEQ ID NO: 1) is less than 5000 nucleotides, however, it can be less than 1000 or even less than 500 nucleotides in length. Preferably, the hybridising polynucleotides are at least 10, more preferably at least 18 nucleotides in length.

In a second aspect, the present invention consists in a genetic construct suitable for use in measuring the induction of a reporter gene, the construct including a nucleic acid molecule according to the first aspect of the present invention operably linked to a nucleic acid molecule encoding a reporter molecule.

Preferably, the nucleic acid molecule encoding the reporter molecule encodes an enzyme. The nucleic acid molecule encoding the reporter may encode the enzyme CYP3A4 or a functionally equivalent enzyme. Examples of suitable reporter molecules include firefly luciferase, beta-galactosidase, chloramphenicol acetyltransferase, alkaline phosphatase and green fluorescent protein. Alternatively, the nucleic acid may encode a messenger RNA (mRNA) that can be detected by standard techniques such as northern blotting or ribonuclease protection assays.

It will be appreciated, however, that the nucleic acid molecule encoding the reporter molecule may be any nucleic acid molecule or gene that, when expressed, has a detectable activity. The nucleic acid molecule

encoding reporter molecule does not have to be associated with the cytochrome P450 system although the inducer of transcription (XREM) used is derived from that gene system. It will also be appreciated that the nucleic acid molecule encoding the reporter gene may include more than one
5 reporter molecule.

In a third aspect, the present invention consists in an assay system including the construct according to the second aspect of the present invention such that on exposure of the system to a xenobiotic inducer, expression of the nucleic acid molecule encoding the reporter molecule is
10 enhanced.

The assay system includes within its scope cells containing the construct (XREM) and cell-free systems capable of supporting the function of the construct (XREM). Such cell-free systems typically contain cell extracts, such as nuclear extracts, but not always. The assay system also includes
15 experimental systems that determine binding of compounds to the construct (XREM). These include DNase I footprinting and gel-retardation assays. These assay systems can be used to detect activation of the construct (XREM), but are not as convenient for mass screening of compounds as the reporter gene constructs. Preferably, the enhanced expression results in an
20 increase in the activity of the reporter gene product.

The cell may be any suitable cell including bacterial, plant or animal cells. The construct may exist as a separate genetic entity in the cell or be incorporated into the genome of the cell. Furthermore, the cell may form part of a transgenic animal.

In a fourth aspect, the present invention consists in a method for screening a compound for xenobiotic induction of CYP3A4 expression in a cell, the method including exposing an assay system according to the third aspect of the present invention to the compound and measuring for the induction or the potential for expression of the nucleic acid molecule
30 encoding the reporter molecule.

The method according to the present invention is particularly suitable for screening new therapeutic drugs. Although there may be many potential drugs available at the developmental stage, if a drug induces CYP3A4 expression *in vivo* then its suitability as an effective therapeutic agent is
35 reduced. In use, the drug's half life will be shorter due to being metabolised by the induced CYP3A4 enzyme in the liver of a patient. Another problem

that can arise is that the drug causes the enhanced metabolism or elimination of other drugs given to the patient. Drug "cocktails" or combinations of drugs are often required to treat many diseases. If one of the drugs administered has the propensity to enhance the clearance of one or more other drugs used in the treatment of a particular disease, then this is highly undesirable and may result in the reduced efficacy of the treatment.

It will be appreciated that if a compound or new drug fails to induce the expression of the reporter gene when tested by the method according to the present invention, then this is an indication that the compound or new drug may not be an xenobiotic inducer and therefore a suitable candidate for further development. A screening process would be beneficial in therapeutic drug development as unsuitable candidates may be disregarded at an early stage of development. Furthermore, alternate or related chemical compounds may also be developed based on a compound's negative result in the screening assay.

The method may also include exposing a transgenic animal and measuring in the animal for induction of the reporter gene after exposure to the chemical or drug.

In a fifth aspect, the present invention consists in the XREM according to the first aspect of the present invention as a genetic analysis tool. In this respect, mutations responding to allelic variants in humans are introduced and their functional consequences observed. Also, as the XREM has a defined function of transcriptional regulation it can be used for the determination of allelic variation.

In a further aspect, the use involves the determination of allelic variation within the XREM (relevant to constitutive expression) and the use of site directed mutation of the XREM to determine the impact of allelic variation.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1 shows a sequence of the XREM region of the CYP3A4 gene. Base numbering is relative and does not indicate location within the CYP3A4 gene structure.

5 Figure 2 shows an example of a series of deletion constructs of the 5'-flanking region of the CYP3A4 gene used to define xenobiotic-responsive elements. Fold induction of luciferase reporter gene expression refers to treatment with a potent CYP3A4 inducer (5 μ M rifampicin) compared to vehicle alone (0.1% dimethyl sulphoxide). This was normalised to the
10 activity of an 'empty' pGL3-basic reporter vector. All constructs were temporarily transfected into HepG2 cells.

Figure 3 shows the effect of cotransfection of a hPXR expression vector (pSG-hPXR) on HepG2 cells containing an CYP3A4 XREM reporter construct, in the presence or absence of a potent CYP3A4 inducer (5 μ M rifampicin).
15 The insert shows a magnified view of the effects on the control cells.

Figure 4 shows the effect of various inducing drugs on HepG2 cells containing a CYP3A4 XREM reporter construct and a hPXR expression vector compared to vehicle (dimethyl sulphoxide) 0.1% alone.. Legend: 1, DMSO; 2, rifampicin; 3, RU-486; 4, clotrimazole; 5, phenobarbital; 6, metyrapone; 7,
20 pregnenalone 16 α -carbonitrile.

Figure 5 shows the effect of deletions and site-directed mutagenesis of putative nuclear receptor response elements on the transcriptional induction of the CYP3A4 5'-flanking region by 5 μ M rifampicin. Filled in boxes correspond to mutated elements. Numbering is relative to the transcription
25 initiation site.

Figure 6 shows an optimised CYP3A4 5'-flanking region construct coupled to a luciferase reporter gene (Luc) for determining the ability of xenobiotics to induce transcriptional activation of the CYP3A4 gene. Numbering is relative to the transcription initiation site.

Modes for Carrying Out the Invention

DEFINITIONS

General Molecular Biology

Unless otherwise indicated, the recombinant DNA techniques utilised
5 in the present invention are standard procedures, well known to those skilled
in the art. Such techniques are described and explained throughout the
literature in sources such as, J. Perbal, A Practical Guide to Molecular
Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning:
A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A.
10 Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes
1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA
Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and
F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene
Pub. Associates and Wiley-Interscience (1988, including all updates until
15 present) and are incorporated herein by reference.

Mutants, Variants and Homology - Nucleic Acids

Mutant polynucleotides will possess one or more mutations which are
deletions, insertions, or substitutions of nucleotide residues. Mutants can be
either naturally occurring (that is to say, isolated from a natural source) or
20 synthetic (for example, by performing site-directed mutagenesis on the DNA).
It is thus apparent that polynucleotides of the invention can be either
naturally occurring or recombinant (that is to say prepared using
recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an
25 individual organism.

Nucleotide sequences are homologous if they are related by divergence
from a common ancestor. Consequently, a species homologue of the
polynucleotide will be the equivalent polynucleotide which occurs naturally
in another species. Within any one species a homologue may exist as
30 numerous allelic variants, and these will be considered homologues of the
polynucleotide. Allelic variants and species homologues can be obtained by
following standard techniques known to those skilled in the art. Preferred
species homologues include those obtained from representatives of the same
Phylum, more preferably the same Class and even more preferably the same
35 Order.

A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) *Ad. Appl. Math.*, 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) *J. Mol. Biol.*, 48: 443-453), to the
5 that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

Substantially Purified

10 By "substantially purified" the present inventors mean a polynucleotide that has been separated from lipids, nucleic acids, other polypeptides or polynucleotides, and other contaminating molecules.

Active Fragment

15 By "active fragment" the present inventors mean a fragment of a sequence shown in Figure 1 which encodes a transcriptional enhancer of cytochrome P450 (P450) termed CYP3A4

BACKGROUND

Human cytochrome P450 3A4 (CYP3A4)

20 CYP3A4 is an important gene in human clinical pharmacology. In addition to it's pivotal role in the metabolism of therapeutic drugs and endogenous steroid hormones, recent studies have associated a non-coding polymorphisms in a putative regulatory region of this gene with both prostate cancer and the development of secondary leukaemia after cancer chemotherapy. An understanding of CYP3A4 gene regulation is important in
25 the development of therapeutic drugs. In addition, CYP3A4 gene represents a novel target for pharmacological manipulation and a tool for both pharmacogenetic analysis and possibly disease prediction. However, a thorough understanding of CYP3A4 regulation and genetics is required to achieve these goals. This present invention is directed to a regulatory module
30 within the 5'-flanking region of the CYP3A4 gene that is responsible for it's xenobiotic induction and constitutive expression.

Human hepatic cytochromes P450s

35 The human hepatic P450s are membrane bound haemoproteins active in the metabolism of a broad range of lipophilic substrates. P450 proteins are of considerable relevance to medicine, not only because of their central role in drug and other xenobiotic metabolism, but also because of their many

'natural' or 'endogenous' lipophilic substrates. These include steroid hormones, fatty acids (especially leukotrienes), cholesterol, and bile salts. It is considered likely that the P450 system evolved because of its pivotal role in cholesterol and steroid metabolism. A small subset of constitutively expressed human hepatic P450s, including CYP3A4, are of particular interest because they are quantitatively the most important forms and catalyse a range of biologically important reactions.

Physiological role and variability of CYP3A4

CYP3A4, the predominant cytochrome P450 found in human liver, comprises up to 60% of the total amount of hepatic cytochrome P450 protein. CYP3A4 is involved in the metabolism of an extensive range of drugs and endogenous steroids. It has been estimated that in excess of 60% of all therapeutic drugs are metabolised in full or in part by this enzyme. Changes in the level of expression of CYP3A4 can have a dramatic impact on the metabolism of therapeutic drugs and thus cause a number of important drug interactions. Also, the variable level of constitutive expression of this enzyme makes a major contribution to the inter-individual variability of oxidative drug metabolism. In addition, CYP3A4 is the major pathway of oxidative metabolism of steroid hormones, catalysing the 6 β -hydroxylation of several endogenous steroids such as cortisol, androstenedione and testosterone as well as the 2- and 4-hydroxylation of estradiol to form catachol estrogens. Two other CYP3A subfamily cytochromes P450 are found in man. CYP3A7 is mainly found in foetal liver while CYP3A5 is found mainly in adult liver as well as the lungs and kidneys.

Regulation of CYP3A4 gene expression

CYP3A4 is subject to multiple levels of transcriptional regulation. These include xenobiotic induction (for example, by some therapeutic drugs), tissue-specific regulation, as well as substantial variability in constitutive expression (up to 10-fold between individuals not exposed to known inducers or inhibitors).

Xenobiotic induction of CYP3A genes

CYP3A genes in several mammalian species, including man, are transcriptionally-inducible by a number of structurally dissimilar therapeutic drugs. Working on the assumption that xenobiotic induction of CYP3A4 is mediated by a response element in the 5'-flanking region of this gene, the present inventors went on to clone 22 kilobases (kb) of the CYP3A4 gene 5' to

the transcription initiation site. This was used to generate a deletion series of constructs covering 13kb of the 5'-flanking region that were linked to a luciferase reporter gene for analysis of transcriptional activity. These constructs were temporarily transfected into a human hepatoblastoma cell line (HepG2) as well as primary cultures of rat and rabbit hepatocytes. By comparing transfected cells treated with inducing agents, such as rifampicin, to untreated controls, the present inventors have determined that the response element [termed hereafter a xenobiotic-responsive enhancer module (XREM)] is approximately located between -7.2kb and -7.8kb 5' to the transcription initiation site (Figure 2). The present studies show the XREM to be a complex element spanning several hundred base pairs (Figure 1, SEQ ID NO: 1). DNase I footprinting further shows that this region is rich in DNA-protein interactions and appears capable of binding several transcription factors of which the recently described human pregnane-X receptor (hPXR) (the pregnane-X receptor is identical to the pregnane-activated receptor (PAR) and the steroid-X receptor (SXR)) [1-3] is the most important for xenobiotic-induced regulation of CYP3A4.

The present inventors have demonstrated that the hPXR forms the vital link between the xenobiotic inducer (eg, rifampicin) and elements within the XREM. In humans, rifampicin treatment typically induces the expression of the CYP3A4 gene 50-fold or more. When a suitable reporter construct containing the XREM sequence is transfected into HepG2 cells, rifampicin treatment results in a 3- to 5-fold induction of reporter gene expression. However, the present inventors have demonstrated that co-transfection of the XREM-containing reporter gene construct with a hPXR expression vector restores full rifampicin induction (Figure 3). The present inventors have also shown that other drugs, such as phenobarbitone and clotrimazole also act on the XREM via the hPXR (Figure 4), demonstrating that this is a general mechanism for xenobiotic induction of the CYP3A4 gene.

The hPXR is an orphan nuclear receptor, belonging to the nuclear receptor gene superfamily [4]. Studies from other laboratories suggest that it binds to DNA as a heterodimer with the retinoid-X receptor (RXR) [1-3]. The present inventors have identified several putative nuclear receptor response elements within the XREM arranged as direct repeats with a 3 base spacer (DR3), direct repeats with a 6 base spacer (DR6) or everted repeats with a 6 base spacer (ER6) (Table 1). In order to evaluate the affinity of these putative

response elements for hPXR-RXR heterodimers, electromobility shift assays (EMSA) were performed using with *in-vitro* transcribed/translated hPXR and RXR. These showed that XREM-DR3-1 and Prox-ER6 efficiently bind hPXR-RXR heterodimers resulting in a gel-shift, but no shift was apparent with XREM-DR6 and XREM-DR3-2. Despite this result, the present inventors have shown by site-directed mutagenesis experiments that the latter two response are functionally important (Figure 5). The XREM-ER6 did bind hPXR-RXR heterodimers, but with less affinity than the XREM-DR3-1 or Prox-ER6.

Table 1. Sequences of putative nuclear receptor response elements within the CYP3A4 XREM compared to a putative response element in the proximal CYP3A4 5'-flanking region (Prox-ER6). An element from the proximal 5'-flanking region of the rat CYP3A23 gene (CYP3A23 DR3) that interacts with the pregnane-X receptor is shown for comparative purposes.

Designation	Sequence (core motifs in bold)	SEQ ID NO:
XREM-DR3-1	gaa TGAACT tgc TGACCC tct	2
XREM-ER6	cct TGAAAT catgtc GGTTCA agc	3
XREM-DR6	agg TGAATC acaagc TGAACT tct	4
XREM-DR3-2	ata TATTGT tat TGAACT atc	5
Prox-ER6	ata TGAACT caaagg AGGTCA gtg	6
CYP3A23 DR3	aga TGAACT tca TGAACT gtc	7

When the XREM is deleted from reporter gene constructs containing the CYP3A4 5'-flanking region, no xenobiotic-inducible activity is seen, demonstrating that the XREM region is essential for the process of xenobiotic induction. Moreover, when the XREM is linked to a heterologous gene promoter, such as a minimal herpes simplex virus thymidine kinase promoter, xenobiotic induction is observed, demonstrating that the XREM is not absolutely reliant on other elements within the CYP3A4 gene. However, for maximal xenobiotic responsiveness, the present inventors have determined that an ER6 element within the proximal promoter region of the CYP3A4 gene (Prox-ER6, Table 1) is required. It is important to appreciate that the Prox-ER6 element has no xenobiotic-inducible activity in the absence of the XREM.

From the above findings, an optimised DNA sequence for the analysis of the xenobiotic induction of the CYP3A4 gene has been determined. It includes the XREM region as well as bases -356 to +53 of the proximal CYP3A4 promoter and is termed construct #5 (Figure 6). Typically, this DNA sequence is linked to a reporter gene and studied in a suitable cell or cell-free system. The present inventors have determined that the HepG2 cell line cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum, transfected using a commercially available reagent (FuGene-6, Boehringer Mannheim, Mannheim, Germany) performs well.

Tissue-specific and constitutive expression of CYP3A subfamily genes

The mechanisms determining the tissue-restricted and constitutive expression of CYP3A4 are probably similar, if not the same. Understanding these aspects of gene regulation are critical to a definitive explanation of why humans exhibit such marked inter-individual variability in CYP3A4-mediated drug metabolism.

Human CYP3A subfamily P450s are predominantly expressed in the liver. However, there is significant tissue-restricted extrahepatic expression. CYP3A4 is expressed in significant amounts in small bowel, colon and pancreas, as well as in breast tissue. The relative levels of constitutive expression in the liver and intestine show little concordance, suggesting that different mechanisms operate in each tissue. While CYP3A4 is constitutively expressed in all adult human livers, there is a 10-fold variability of CYP3A4 mRNA between liver samples. Variability in intestinal expression is even more marked, with > 30-fold variation being reported.

In the case of hepatic CYP3A4 expression, using reporter constructs containing the 5'-flanking region of the CYP3A4 gene, the present inventors have demonstrated that liver-specific factors are preferably required to support CYP3A4 transcription. When a liver-derived cell line (HepG2) was compared to a non-liver-derived line (NIH-3T3) for their ability to support transcriptional activation, either in the presence or absence of hPXR, the non-liver-derived cell line was markedly inferior. Clearly, this suggests an optional role for liver-specific transcription factors. However, the present inventors know that HepG2 cells, despite being a human liver-derived cell line, do not constitutively express CYP3A4 in significant amounts. The present inventors have found that an unusual human orphan nuclear receptor, termed the human constitutive androstane receptor- β (hCAR- β) [5],

when cotransfected into HepG2 cells with a CYP3A4 5'-flanking region reporter construct, causes a 6- to 10-fold increase in reporter activity. hCAR- β requires no ligand to transactivate regulatory DNA sequences and its expression is almost entirely restricted to the liver. Experiments performed using deletion constructs show that the response of the CYP3A4 gene to the hCAR- β receptor is dependant on the XREM. Also, as observed for xenobiotic induction with the hPXR, hCAR- β -mediated constitutive expression was observed when the XREM was linked to the minimal thymidine kinase promoter, though at a lower level than observed with the native promoter. Preliminary experiments suggest that there is cooperativity between the XREM and the Prox-ER6 element in hCAR- β -mediated constitutive expression. However, the native promoter alone shows no response to hCAR- β cotransfection.

RESULTS

Cytochrome P450 3A4 (CYP3A4), the predominant P450 expressed in adult human liver, is subject to transcriptional induction by variety of structurally unrelated xenobiotic compounds, including the antibiotic rifampicin. The present inventors have transfected a human liver-derived cell line (HepG2) with various CYP3A4-luciferase reporter gene constructs containing a nested set of 5'-deletions of the CYP3A4 promoter. Rifampicin-inducible transcription of the reporter gene was only observed with the longest construct. Rifampicin treatment of cells transfected with the -13000/+53-luciferase construct resulted in a 3- to 5 fold increase in reporter gene activity. This construct was activated in a dose-dependent manner by rifampicin with maximal induction at 5 μ M. A further set of deletion clones were prepared and the responsive region localised to bases -7800 to -7200, approximately. Polymerase chain reaction-generated deletion mutants suggest that rather than being dependent on a short *cis*-acting element, the rifampicin-response is reliant upon the integrity of larger region encompassing several hundred bases. This region, in conjunction with a minimal CYP3A4 promoter (-362 to +53), was capable of conferring rifampicin-responsiveness on the reporter gene. The induction was independent of the orientation of the -7800/-7200 fragment and its position relative to the proximal promoter of CYP3A4. Heterologous reporter gene constructs, containing the distal enhancer region of CYP3A4 ligated to the herpes simplex virus thymidine kinase promoter, were also capable of

inducing luciferase expression following rifampicin treatment. Nucleotide sequence analysis of this region revealed a number of putative transcription factor binding sites. In summary, the present inventors have identified an enhancer region in the CYP3A4 gene capable of mediating transcriptional activation by rifampicin.

Using a series of deletion constructs of the CYP3A4 gene 5'-flanking region, the present inventors have discovered a DNA sequence, lying approximately 7.5 kilobases 5' to the transcription initiation site of the human P450 CYP3A4 gene, that is responsible for the transcriptional induction of the CYP3A4 gene by xenobiotic inducers, including therapeutic drugs. The present inventors have called this element a 'xenobiotic-responsive enhancer module' (XREM). This work has initially performed with the potent CYP3A4 inducer, rifampicin, an antibiotic commonly used to treat tuberculosis. Preliminary studies with other drugs, however, suggest that the XREM may be capable of responding to a broad range of chemical compounds. The present inventors have used the XREM-containing DNA sequence from CYP3A4 to construct cell culture models that respond to xenobiotic inducers by increasing the transcription of the CYP3A4 gene in a manner analogous to that occurring in humans.

The present inventors cotransfected a CYP3A4 5'-flanking region construct, containing the XREM and linked to a luciferase reporter, into HepG2 cells either with or without a hPXR expression construct (pSG-hPXR). The addition of the hPXR substantially increased the transcription rate of the reporter construct, both constitutively (treated with vehicle alone) and following the addition of a xenobiotic inducer (5 μ M rifampicin). The observation that addition of the hPXR alone increases the transcription rate of the CYP3A4 XREM-containing reporter indicates that the hPXR is capable of some transactivation in the absence of ligand or that an endogenous ligand for the hPXR exists within the HepG2 cells.

The hPXR has been shown to bind to cis-acting DNA response elements (PXRE) as a heterodimer with the retinoid-x receptor (RXR). The PXRE seems to generally consist of a repeat of the sequence TGAAC, either as direct or everted/inverted repeats. The present inventors have identified a number of putative nuclear receptor response elements (PXREs) within the XREM shown in Table 1.

The present inventors have performed gel mobility shift experiments with ^{32}P -labelled oligonucleotide probes to determine if hPXR-RXR heterodimers were capable of binding to the putative PXREs within the XREM.

5 To further examine the contribution of the putative PXREs within the XREM region to the transcriptional induction of CYP3A4 by rifampicin, a series of luciferase reporter constructs containing deletions and/or site-directed mutagenesis of the PXREs were created (Figure 5). Deletion of the region containing the PXRE XREM-DR3-1 (-7834 to -7610 bp) resulted in
10 almost complete abrogation of transcriptional activity. This was not entirely due to the loss of function of XREM-DR3-1 as site directed mutagenesis of this PXRE resulted in only a 47% loss of activity. Mutation of XREM-DR6 also decreased transcription rate (to 52% of the wild-type) despite the apparent failure of this motif to bind a hPXR-RXR heterodimer in the gel-
15 shift experiment. It is possible that this site binds other transcription factors or is a low-affinity site for hPXR-RXR.

It can be appreciated that the putative PXRE within the proximal promoter region which consists of the Prox-ER6 motif has no function on its own. However, site-directed mutagenesis of the Prox-ER6 in a construct
20 containing the complete XREM does decrease the transcription rate (to 41% of the wild-type) (Figure 5) suggesting cooperativity between the XREM and Prox-ER6.

The XREM mediates the transcriptional induction of the CYP3A4 gene to many xenobiotics, not only rifampicin

25 Many drugs induce the transcription of the CYP3A4 gene. Because of its potent induction properties, the present inventors used rifampicin to identify and characterise the XREM. To determine if an XREM-containing reporter construct was capable of responding to other xenobiotics, HepG2 cells transfected with the XREM were treated with a range of drugs (Figure
30 4). It was found that RU-486 (mifaprostone) and clotrimazole induced the XREM construct in a manner analogous to that that occurs *in vivo*. The present inventors have also examined a wider range of drugs known to induce CYP3A4 *in vivo* and found that the *in vitro* model developed closely reflects *in vivo* experience.

USES FOR THE INVENTION

The invention has several areas of application, including but not restricted to, therapeutic drug development. In some of these areas of application, XREM containing DNA constructs are used to determine the effects of compounds on CYP3A gene transcription. These constructs typically include reporter genes to allow for the convenient measurement of gene activity. Such reporter genes include chloramphenicol acetyl transferase, luciferase, alkaline phosphatase and green fluorescent protein, though any detectable gene product, including messenger RNA (mRNA) could be used. Such gene constructs are introduced transiently or permanently into suitable cultured cells, or as a transgene in transgenic animals. Additional DNA constructs can be cotransfected along with the XREM-containing constructs to provide suitable conditions to answer particular questions regarding CYP3A gene regulation. Such cotransfected constructs include expression plasmids for hPXR and hCAR- β which the present inventors have demonstrated to interact with the XREM. Present studies have also shown that there are many protein-DNA interactions within the XREM so it is likely that other cotransfected constructs will prove useful for certain situations.

Another area of application is the use of the XREM sequence to examine human genomic DNA for the presence of polymorphisms relevant to CYP3A4 gene expression. Such polymorphisms may be linked to several areas of use including the prediction of drug metabolising ability and disease associations.

The use of cell or transgenic animal models to screen chemical substances for the ability to induce the formation of the CYP3A4 enzyme

Induction of CYP3A subfamily enzymes by therapeutic drugs is generally considered to be an undesirable effect. Such induction may increase the elimination of the drug itself or co-administered drugs. This generally renders drugs less effective. The ability to screen potential drugs for CYP3A induction during the early development phase is a useful in that such drugs may be discarded in favour of ones that do possess this action. The information gained from such drug screening also allows the development of structure-function relationships, which indicate molecules or parts of molecules that have the propensity to induce CYP3A4. Such knowledge allows the use of rational drug design to synthesize new

compounds that have more favourable characteristics. This process can be applied to existing drugs that undesirably induce CYP3As, such as those listed in Table 2.

- 5 Table 2. A non-exclusive list of therapeutic drugs known to induce the expression of the CYP3A4 gene.

Drug
Carbamazapine
Clotrimazole and other imidazole anti-mycotics
Lovastatin
Phenytoin
Phenobarbitone
Rifampacin
Rifabutin
RU-486

- 10 In addition to the above, there are potential therapeutic uses for drugs specifically designed to induce or inhibit CYP3A enzyme formation as their primary action. Inducers of CYP3A4 can be used to accelerate the metabolism of xenobiotic toxins or endogenously produced substances that are CYP3A4 substrates. Inhibitors of CYP3A4 could be used to overcome the undesirable induction of CYP3A4 by therapeutic drugs, such as those listed
- 15 in Table 2. It will be appreciated that there are many potential uses of drugs specifically designed to modulate CYP3A enzyme formation, other than those listed here. The XREM sequence is useful in identifying such drugs.

The XREM as a tool for pharmacogenetic analysis / disease susceptibility

- 20 There are marked inter-individual differences in the capacity of humans to metabolise xenobiotic compounds (such as therapeutic drugs) and endogenous compounds (such as endogenously-secreted hormones). At least some of these differences can be related to polymorphisms in the genes encoding the enzymes or transport proteins that interact with these xenobiotic or endogenously produced compounds.

- 25 Members of the cytochrome P450 gene superfamily are involved in the metabolism of a large range of lipophilic substrates. CYP3A subfamily P450s

(such as CYP3A4) are particularly involved in the metabolism of therapeutic drugs and endogenously produced steroid hormones. It is well recognised that there are marked inter-individual differences in the metabolism of CYP3A substrates (up to 20-fold), however, to date, no polymorphisms within the CYP3A4 protein coding region of the CYP3A4 gene have been found to account for these differences. This strongly suggests that it is the regulation of CYP3A gene expression that accounts for these inter-individual differences.

The present inventors have demonstrated that the XREM region of CYP3A4 is an important regulatory element for the transcriptional control of the expression of this gene. It follows that polymorphisms within the XREM could significantly effect gene transcription and the expression of CYP3A4 protein, thus explaining, at least in part, the inter-individual differences in CYP3A-mediated metabolism.

Rebbeck and colleagues have recently described a polymorphism in the proximal 5'-flanking region of the CYP3A4 gene that correlates with more severe forms of prostate cancer [6] and a reduced incidence of secondary leukaemia following cancer chemotherapy [7]. It is presumed that this polymorphism caused these changes due to effects on CYP3A4 expression though this has yet to be proven.

Thus, use of the XREM sequence and function provides a potentially powerful tool to search for CYP3A gene polymorphisms. Screening for such polymorphisms would be of considerable usefulness in determining the ability of an individual to metabolise drugs (pharmacogenetic analysis) or determine disease susceptibility (eg., prostate cancer). The method for carrying out such screening typically involves the amplification of the genomic DNA region of interest (in this case the XREM) using polymerase chain reaction (PCR). The PCR product can then be examined for polymorphisms using one of several techniques such as restriction fragment length polymorphisms (RFLP), sequencing of DNA or single-stranded conformational polymorphisms (SSCP) of DNA.

The use of the XREM as an inducible enhancer for gene expression in the broader context of molecular biology, transgenics and directed gene expression

5 The concept of using a portion of a cytochrome P450 gene as a genetic switch has been previously demonstrated using the CYP1A1 gene. As the XREM region of the CYP3A4 gene is capable of regulating transcription in response to xenobiotics as well as certain steroid hormones, it is potentially useful as a genetic switch within the broad context of molecular biology and directed gene expression. An example is the activation of a transgene in the
10 liver of an animal via a PXR/XREM-dependant mechanism.

 It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to
15 be considered in all respects as illustrative and not restrictive.

References

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5
2. Bertilsson G, *et al.* (1998) Identification of a human nuclear receptor defines a new pathway for CYP3A induction. *Proc Natl Acad Sci USA* 95:12208-12213.
10
3. Blumberg B, *et al.* (1998) SXR, a novel steroid and xenobiotic sensing receptor. *Genes & Dev* 12:3195-3205.
4. Mangelsdorf DJ, *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell* 83:835-839.
15
5. Baes M, *et al.* (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol Cell Biol* 14:1544-52.
20
6. Rebbeck TR, *et al.* (1998) Modification of the clinical presentation of prostate tumors by a novel genetic variant in CYP3A4. *J Natl Cancer Inst* 90:1225-1229.
7. Felix CA, *et al.* (1998) Association of CYP3A4 genotype with treatment-related leukemia. *Proc Natl Acad Sci USA* 95:13176-81.
25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: University of Sydney
- (B) STREET:
- (C) CITY: Sydney
- (D) STATE: New South Wales
- (E) COUNTRY: Australia
- (F) POSTAL CODE (ZIP): 2006

(ii) TITLE OF INVENTION: Xenobiotic induction of gene expression

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 632 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GGTCATAAAG CCCAGTTTGT AAACATGAGAT GATCTCAGCT GAATGAACTT GCTGACCCCTC 120
TGCTTTCCTC CAGCCTCTCG GTGCCCTTGA AATCATGTCTG GTTCAAGCAG CCTCATGAGG 180
CATTACAAAG TTTAATTATT TCAGTGATTA TTAAACCTTG TCCTGTGTTG ACCCCAGGTG 240
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AGTAAGTTAA TTGATAGGAT TTTTTTTTGT TAAAAAAAAT GTTACTAGTT TTTGAAAAGG 360
TAATATGTTG CACATGGTAA AACTAAGAA GGTATAAGAG CATAATGCTT TTATACTACT 420
AAGAATAATG TTTTCTCTAA GTTTTTTTTTG GTAGATGCTT TCATCAGATT AAGAAAATTC 480
CCTGCTATTA GTTGTTGAAG GTTTTATATAT CATAAATGAA AGTTGAATAT TATTATCATA 540
TATTATTAAT ATATTGTTAT TGAACATCA AAGCCTTTTC CTAAAACCAT TGAGATGATC 600
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22

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATGAACTT GCTGACCCTC T

21

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTTGAAATC ATGTCGGTTC AAGC

24

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGTGAATCA CAAGCTGAAC TTCT

24

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATATGAACTC AAAGGAGGTC AGTG

24

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AGATGAACTT CATGAACTGT C

21

CLAIMS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a transcriptional enhancer of cytochrome P450 (P450) CYP3A4 production or expression.
- 5 2. The nucleic acid molecule according to claim 1 comprising a nucleotide sequence substantially as shown in SEQ ID NO: 1, or a functionally equivalent nucleotide sequence or portion thereof encoding an enhancer of CYP3A4, or a sequence which hybridises to the nucleotide sequence of SEQ ID NO: 1, or a sequence which shows at least 60%
10 homology with the nucleotide sequence of SEQ ID NO: 1.
3. The nucleic acid molecule according to claim 2 having at least 80% homology with the nucleotide sequence of SEQ ID NO: 1.
4. The nucleic acid molecule according to claim 3 having at least 90% homology with the sequence of SEQ ID NO: 1.
- 15 5. The nucleic acid molecule according to claim 2 having a nucleotide sequence substantially as shown in SEQ ID NO: 1.
6. The nucleic acid molecule according to any one of claims 1 to 5 wherein the CYP3A4 is human CYP3A4.
7. An isolated polynucleotide having at least 10, preferably having at
20 least 18 nucleotides, which hybridises to a molecule comprising a nucleotide sequence shown in SEQ ID NO: 1.
8. An isolated nucleic acid molecule including a nuclear receptor response element from the enhancer of CYP3A4 shown in SEQ ID NO: 1.
9. An isolated nucleic acid molecule according to claim 8 including a
25 nuclear receptor response element selected from the group consisting of
GAA TGAAC TTGC TGACCC TCT (SEQ ID NO: 2);
CCT TGAAAT CATGTC GGTTCA AGC (SEQ ID NO: 3);
AGG TGAATC ACAAGC TGAAC TCT (SEQ ID NO: 4);
ATA TATTGT TAT TGAAC ATC (SEQ ID NO: 5); and
30 ATA TGAAC TCAAAGG AGGTCA GTG (SEQ ID NO: 6).
10. A genetic construct suitable for use in measuring induction of a reporter molecule, the construct comprising a nucleic acid molecule according to any one of claims 1 to 9 operably linked to a nucleic acid molecule encoding a reporter molecule.
- 35 11. The construct according to claim 10 wherein the nucleic acid molecule encoding the reporter molecule encodes an enzyme.

12. The construct according to claim 9 wherein the enzyme is selected from the group consisting of firefly luciferase, beta-galactosidase, chloramphenicol acetyltransferase, alkaline phosphatase and green fluorescent protein.
- 5 13. The construct according to claim 10 wherein the reporter molecule is a messenger RNA (mRNA) molecule.
14. An assay system suitable for use in measuring induction of a reporter molecule, the system comprising a construct according to any one of claims 10 to 13 and means to support the function of the construct, such that on
10 exposure of the system to a xenobiotic inducer, expression of the nucleic acid molecule encoding the reporter molecule is enhanced.
15. The system according to claim 14 wherein the means to support the function of the construct includes cells, transgenic non-human mammals and cell-free systems capable of supporting the function of the construct.
- 15 16. The system according to claim 15 wherein the cell is selected from the group consisting of bacterial, plant, and animal cells.
17. The system according to claim 16 wherein the construct resides in the cell as a separate genetic entity or incorporated into the genome of the cell.
18. A method of screening a compound for xenobiotic induction of
20 CYP3A4 expression in a cell, the method comprising exposing an assay system according to any one of claims 14 to 17 to the compound and measuring for the induction or the potential for expression of the nucleic acid molecule encoding the reporter molecule of the construct.
19. The method according to claim 18 wherein the compound is a
25 therapeutic drug.
20. Use of the isolated nucleic acid molecule according to any one of claims 1 to 9 as a genetic analysis tool.
21. The use according to claim 20 selected from the group consisting of determination of allelic variants within SEQ ID NO: 1 for predicting drug
30 metabolism or disease susceptibility, and analysing effects of allelic variants on CYP3A4 transcription and expression.

TCTAGAGAGA TGGTTCATTC CTTTCATTTG ATTATCAAAG AACTCATGT CCCAATTAAA 60
GGTCATAAAG CCCAGTTTGT AACTGAGAT GATCTCAGCT GAATGAACTT GCTGACCCTC 120
TGCTTTCCTC CAGCCTCTCG GTGCCCTTGA AATCATGTCG GTTCAAGCAG CCTCATGAGG 180
CATTACAAAG TTTAATTATT TCAGTGATTA TTAAACCTTG TCCTGTGTTG ACCCCAGGTG 240
AATCACAAGC TGAACCTCTG ACAAGAACAA GCTATCATAT TCTTTTCAAT TACAGAAAAA 300
AGTAAGTTAA TTGATAGGAT TTTTTTTGTT TAAAAAAAT GTTACTAGTT TTTGAAAAGG 360
TAATATGTTG CACATGGTAA AACTAAGAA GGTATAAGAG CATAATGCTT TTATACTACT 420
AAGAATAATG TTTTCTCTAA GTTTTTTTTG GTAGATGCTT TCATCAGATT AAGAAAATTC 480
CCTGCTATTA GTTGTGAAG GTTTTATAT CATAAATGAA AGTTGAATAT TATTATCATA 540
TATTATTAAT ATATTGTTAT TGAACATCA AAGCCTTTTC CTAAAACCAT TGAGATGATC 600
TTATAACCAT TCTCCTTTAA CCTGTTGACG AG 632

Figure 1

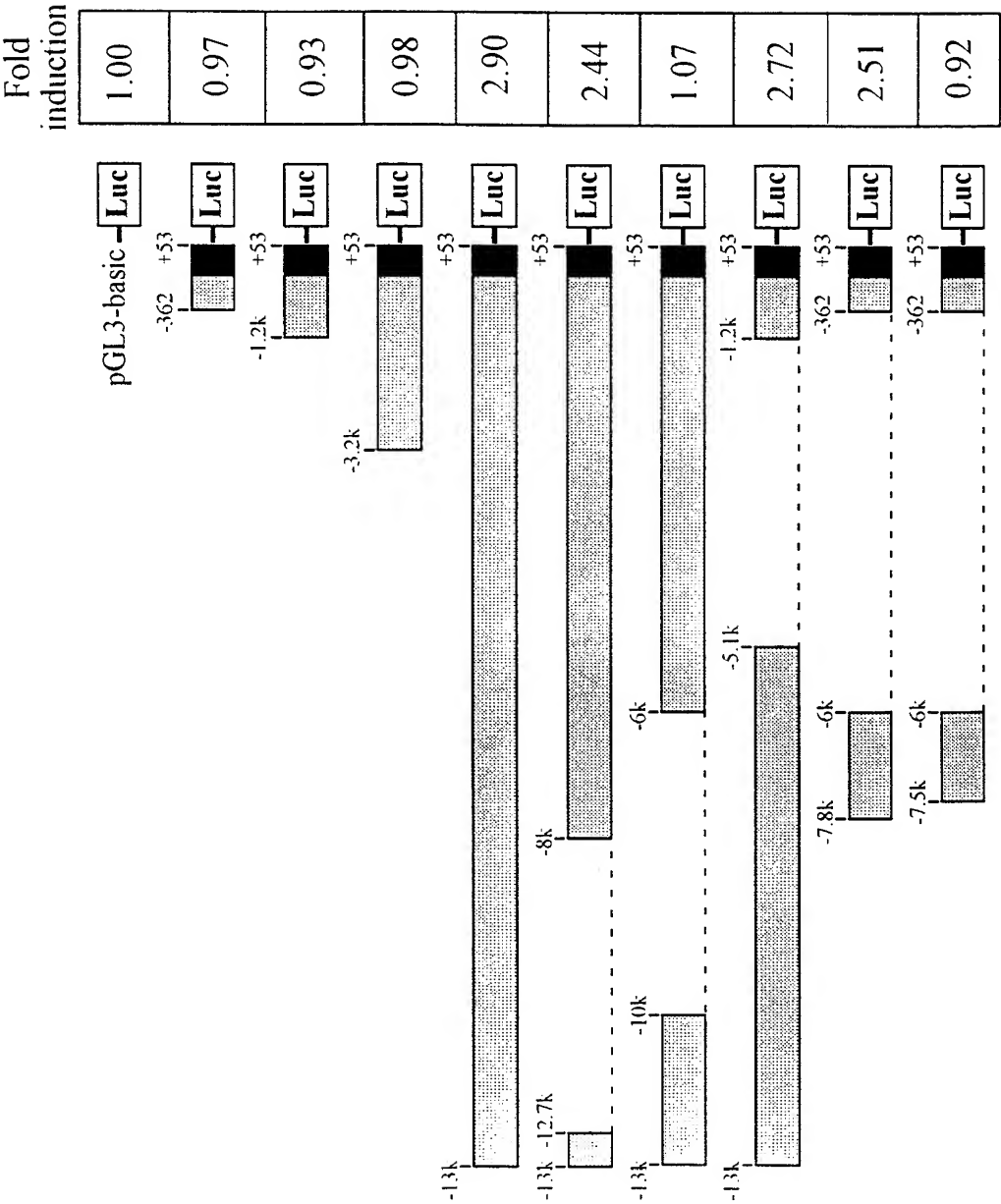


Figure 2

3/6

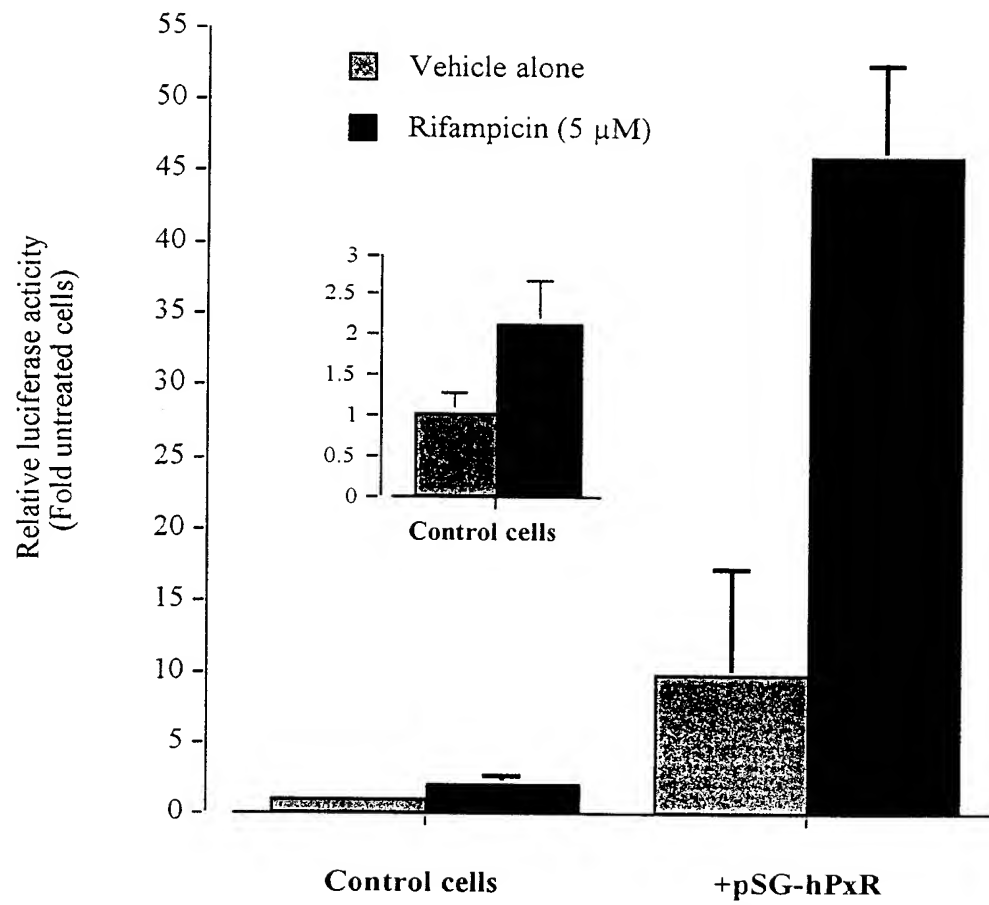


Figure 3

4/6

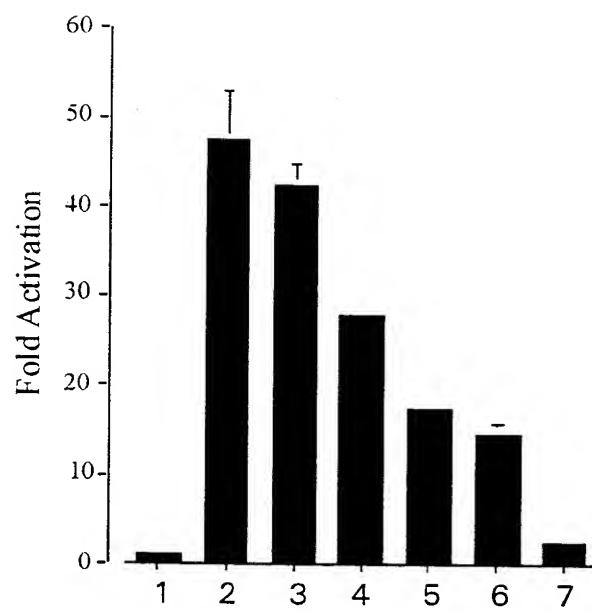


Figure 4

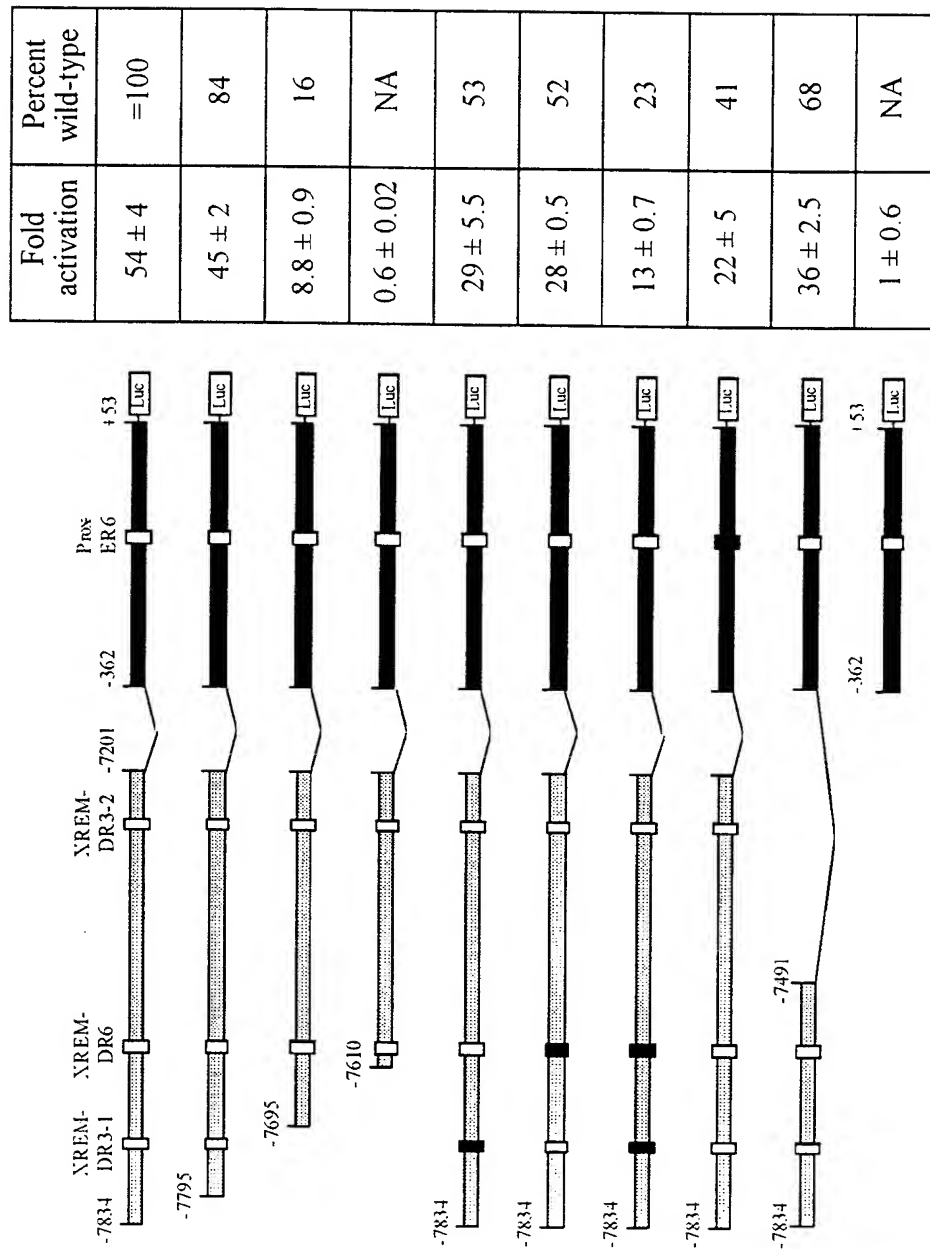


Figure 5

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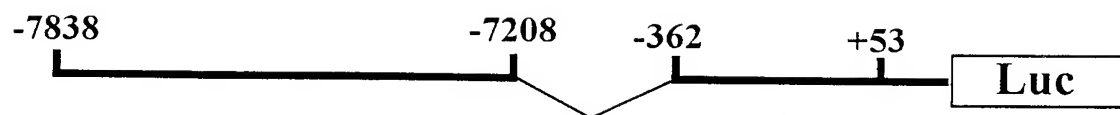


Figure 6

1/3

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: University of Sydney
- (B) STREET:
- (C) CITY: Sydney
- (D) STATE: New South Wales
- (E) COUNTRY: Australia
- (F) POSTAL CODE (ZIP): 2006

(ii) TITLE OF INVENTION: Xenobiotic induction of gene expression

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

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- (A) LENGTH: 632 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTAGAGAGA TGGTTCATTC CTTTCATTTG ATTATCAAAG AAACATCATGT CCCAATTAAA 60
GGTCATAAAG CCCAGTTTGT AAACATGAGAT GATCTCAGCT GAATGAACTT GCTGACCCCTC 120
TGCTTTCCTC CAGCCTCTCG GTGCCCTTGA AATCATGTCG GTTCAAGCAG CCTCATGAGG 180
CATTACAAAG TTTAATTATT TCAGTGATTA TTAAACCTTG TCCTGTGTTG ACCCCAGGTG 240
AATCACAAGC TGAACCTCTG ACAAGAACAA GCTATCATAT TCTTTTCAAT TACAGAAAAA 300
AGTAAGTTAA TTGATAGGAT TTTTTTTGTT TAAAAAAAAT GTTACTAGTT TTTGAAAAGG 360
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CCTGCTATTA GTTGTTGAAG GTTTTTATAT CATAAATGAA AGTTGAATAT TATTATCATA 540
TATTATTAAT ATATTGTTAT TGAACATCA AAGCCTTTTC CTAAAACCAT TGAGATGATC 600
TTATAACCAT TCTCCTTTAA CCTGTTGACG AG 632

2/3

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATGAACTT GCTGACCCTC T

21

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTTGAAATC ATGTCGGTTC AAGC

24

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGGTGAATCA CAAGCTGAAC TTCT

24

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

3/3

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
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- (2) INFORMATION FOR SEQ ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
ATATGAACTC AAAGGAGGTC AGTG 24
- (2) INFORMATION FOR SEQ ID NO: 7:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
AGATGAACTT CATGAACTGT C 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00381

A. CLASSIFICATION OF SUBJECT MATTER												
Int Cl ⁶ : C12N 15/52												
According to International Patent Classification (IPC) or to both national classification and IPC												
B. FIELDS SEARCHED												
Minimum documentation searched (classification system followed by classification symbols) as below												
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched as below												
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) ORBIT (WPAT): (cytochrome P450) and C12N-015/IC STN (Medline): (cytochrome P450) and CYP3? Genbank: SEQ. I.D. Nos 1-6												
C. DOCUMENTS CONSIDERED TO BE RELEVANT												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X,Y	Eur. J. Biochem, <u>218</u> , pp 585-595 (1993) Hashimoto et al; see p587, 588	1-21										
X,Y	Clinical and Experimental Pharmacology and Physiology, <u>25</u> , pp 1-9 (Jan, 1998). Dorga et al.	1-21										
X	Pharmacogenetics, <u>4</u> , 247-259 (1994) Kolars et al.	1-21										
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex												
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td>"&" document member of the same patent family</td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
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Date of the actual completion of the international search 1 July 1999		Date of mailing of the international search report 20 JUL 1999										
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer MADHU K. JOGIA Telephone No.: (02) 6283 2512										

INTERNATIONAL SEARCH REPORT

international application No.

PCT/AU 99/00381

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications, <u>221</u> , pp129-132 (1996). Kovaleva et al.	1-21
X	European J of Drug Metabolism and Pharmacokinetics, Vol 22 (4), pp 311-313 (1997) Ogg et al.	14-19
X	Critical Reviews in Toxicology, <u>28</u> (1), pp 35-72 (Jan, 1998). Makkota et al.	14-19
X	<u>Mol. Cell. Biol.</u> , <u>10</u> , pp 1470-1475 (1990) Gonzalez et al. and <u>DNA</u> , <u>7</u> , 79-86 (1988), and Genbank Accession No D11131.	9
X	US 5429948 (Davies et al) 4 July 1995	1-21
X	EP 644267 (Sumitoto Chem Co Ltd) 22 March 1995	1-21

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Information on patent family members

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PCT/AU 99/00381

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
EP	644267	CA	2128399				
US	5429948	AU	88781/91	EP	555315	WO	9207085
							END OF ANNEX